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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<p>(21) International Application Number: <b>PCT/US96/08619</b> (22) International Filing Date: <b>6 June 1996 (06.06.96)</b>  (30) Priority Data: 08/483,090      7 June 1995 (07.06.95)      US  (71) Applicant: <b>GEORGETOWN UNIVERSITY [US/US]; Ryan Administration Building, 37th and O Streets, N.W., Washington, DC 20057 (US).</b>  (72) Inventors: <b>THIERRY, Alain; Apartment #501, 4970 Battery Lane, Bethesda, MD 20814 (US). DRITSCHLO, Anatoly; 8101 Fenway Road, Bethesda, MD 20817 (US).</b>  (74) Agents: <b>BENT, Stephen, A. et al.; Foley &amp; Lardner, Suite 500, 3000 K Street N.W., Washington, DC 20007-5109 (US).</b></p>		<p>(81) Designated States: <b>AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).</b>  <b>Published</b> <i>With international search report.</i></p>
<p>(54) Title: <b>A METHOD OF TRANSFECTION OF CELLS USING LIPOSOMALLY ENCAPSULATED NUCLEIC ACIDS</b>  (57) Abstract  An improved method for encapsulating high molecular weight nucleic acids in liposomes, which provides for high nucleic acid entrapment efficiencies, is provided. The resulting compositions provide enhanced <i>in vitro</i> and <i>in vivo</i> transfection and are useful, for example, in producing cell lines expressing a desired nucleic acid sequence.</p>		

A METHOD OF TRANSFECTION OF CELLS  
USING LIPOSOMALLY ENCAPSULATED NUCLEIC ACIDS

BACKGROUND OF THE INVENTION

5        Cells that express heterologous gene products are valuable as research tools, as "factories" for the production of gene products, and for gene therapy. It has therefore become increasingly important to be able to custom engineer cells with heterologous polynucleotide  
10       constructs rapidly and inexpensively. A major factor in the time and cost of generating such cells lines is the efficiency with which polynucleotides can be introduced and stably incorporated into the desired cells.

15       Currently, a variety of methods exist for transfecting polynucleotides into target cells. Such methods include conventional means such as calcium phosphate and polycation-mediated transfection, as well as protoplast fusion, viral and retroviral infection, microinjection and electroporation.

20       Another method recently investigated for delivery of polynucleotides into cells involves the administration of liposomally-encapsulated nucleic acids. Such methods originated in the early 1980's when Papahadjopoulos et al. disclosed the encapsulation of biologically-active  
25       materials, such as nucleic acids and proteins, in liposomes. These liposomes were then used for delivery of the biologically active materials to target cells. See, e.g., U.S. Patent No. 4,241,046; U.S. Patent No. 4,235,871; U.S. Patent No. 4,394,448. See also, Lasic et al., *Science* 267:1275 (1995).  
30

      Such methods have been improved in order to enhance efficiency of the transfection process. For example, conjugating liposomes to ligands which selectively bind to targeted cells is known to enhance the efficiency of  
35       delivery to desired cells. Relevant patents which disclose the conjugation of liposomes to cell-targeting moieties, in particular, to antibodies, are e.g., U.S. Patent No. 5,210,040; U.S. Patent No. 4,957,735; U.S.

teaches liponucleotide containing liposomes and the use thereof in delivery of the liponucleotides to desired cells.

Thus, it is clear that many methods for delivery of nucleic acids into cells are known in the art, as are methods which rely on liposomally-mediated introduction of nucleic acids. Conventional liposome encapsulation methods are subject to many constraints. For example, conventional methods for encapsulation of nucleic acids into liposomes are limited by poor entrapment efficiencies. That is, only a small percentage of the initial nucleic acid used actually becomes encapsulated. As discussed supra this is undesirable because it is inefficient both with respect to its use of DNA and with respect to the quantity of liposomes that must be used to deliver a given amount of DNA.

Also, most conventional methods for liposome encapsulation of nucleic acids are suitable only for encapsulation of small nucleotides, e.g., oligonucleotides. This is disadvantageous since expression of heterologous gene products in cells often requires the introduction of much larger DNA sequences that include not only coding regions, but also other *cis*-acting controls for regulating gene expression, such as promoter and enhancer sequences, operator sequences and the like, as well as a ribosome binding site, an initiation codon and transcription termination and polyadenylation signals. Buffer regions, origins of replication for extra-chromosomal replication, and flanking regions with homology to a target site for targeted chromosomal insertion by homologous recombination may also be included.

Present methods do not permit the efficient encapsulation of large DNA vectors such as plasmids and phagemids. This is particularly disadvantageous for those vectors that are designed to be maintained in an episomal state, i.e., extrachromosomally. These vectors can avoid the potential problems which occur during non-

lipid film for an effective time at reduced temperature, where the hydrated lipid film is formed by addition of a concentrated aqueous solution of a high molecular weight nucleic acid to a dried lipid film; (ii) adding a minimum  
5 effective amount of phosphate buffered saline solution to the hydrated lipid film and vortexing for a time sufficient to generate liposomes and to swell the liposomes; and (iii) vortexing the resulting swelled liposomes. The method may also further comprise the step  
10 of (iv) removing unencapsulated nucleic acid from said liposomes by washing said liposomes. The washing may be achieved by centrifugation.

In accordance with another aspect of the invention, the lipid film comprises at least one lipid selected from  
15 the group consisting of dimyristoyl-diglycerol, phosphatidylethanolamine, phosphatidylcholine, phosphatidic acid, lysophosphatidylcholine, phosphatidylserine, sphingolipids, phosphatidylglycerol, sphingomyelin, cardiolipin, glycolipids, gangliosides, cerebrosides, cholesterol, tocopherol, and retinol.  
20

In accordance with a further aspect of the invention the lipid film comprises dimyristoyldiglycerol, phosphatidylethanolamine and cholesterol, in a relative molar ratio of about 5:5:7 respectively.

25 In accordance with yet another aspect of the invention the nucleic acid solution has a concentration of 1-5 mg/ml, and the nucleic acid solution is added to the dried lipid film in a ratio of less than or equal to about 1.6  $\mu$ l per mg of lipid. The effective time for the  
30 incubating step is preferably at least 12 hours, and the time for forming and swelling the liposomes is at least 30 minutes.

In accordance with still another aspect of the invention the size of the nucleic acid is preferably from  
35 about 1 kB to about 25 kB, or more preferably from about 5 kB to about 18 kB.

In accordance with a still further aspect of the invention there has been provided a method for

described above, wherein the therapeutic protein is selected from the group consisting of platelet-derived growth factor, epidermal growth factor, interleukins 1-14, granulocyte colony stimulating factor, granulocyte-macrophage colony stimulating factor, tumor necrosis factor, leukemia inhibitory factor, amphiregulin, angiogenin, betacellulin, calcitonin, ciliary neurotrophic factor, brain-derived neurotrophic factor, neurotrophins 3 and 4, nerve growth factor, colony stimulating factor-1, endothelial cell growth factor, erythropoietin, acidic and basic, fibroblast growth factor, hepatocyte growth factor, heparin binding EGF-like growth factor, insulin, insulin-like growth factors I and II, interferons  $\alpha$ ,  $\delta$ , and  $\gamma$ , keratinocyte growth factor, macrophage inflammatory protein  $\alpha$  and  $\delta$ , midkine, oncostatin M, RANTES, stem cell factor, transforming growth factors  $\alpha$  and  $\delta$ , and vascular endothelial growth factor.

#### DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention provides an improved method for the encapsulation of high molecular weight nucleic acids in liposomes, wherein a higher nucleic acid entrapment efficiency is achieved than can be achieved by conventional methods for encapsulating nucleic acids in liposomes. In the present invention, entrapment efficiency is defined as the percentage of the initial amount of the nucleic acid used which becomes encapsulated in the resulting liposomes.

This improved method also provides liposomal compositions in which a greater proportion of the liposomes actually contain the desired nucleic acids, than can be achieved with conventional methods for encapsulating nucleic acids in liposomes. Additionally, each liposome comprises a higher nucleic acid content, on average, than liposomes generated by conventional techniques.

These characteristics are advantageous for several reasons. First, the high encapsulation efficiency, which

sequences, operator sequences and the like, and the polynucleotide also contains a ribosome binding site, an initiation codon and transcription termination and polyadenylation signals. The definition of HMW  
5 polynucleotides as used herein is, therefore, generally understood to mean polynucleotides that contain such regulatory elements. The HMW polynucleotide may also contain other elements such as origins of replication as are commonly found on polynucleotides used for  
10 transfection.

The present invention provides for the efficient encapsulation of large vectors, including those which contain sequences that permit stable, episomal maintenance and those which encode multigene cassettes.  
15 This is significant, in the case of episomal constructs, because integration of the desired nucleic acid into the host cell's genome may have a negative impact on the transfection process. For multigene cassettes, it also is important as coordinate regulation of the encoded  
20 genes can be more easily achieved.

The nucleic acids which may be encapsulated according to the present method may range in size from as small as about 500 bases to about 50 kilobases. In a preferred embodiment, the encapsulated nucleic acids will comprise  
25 DNA's ranging from about 1.0 to 25 kB and, preferably, from about 5 to about 18 kB.

The nucleic acids which may be encapsulated according to the present method may comprise sense or antisense polynucleotides. For example, antisense oligonucleotides  
30 may be encapsulated which selectively inhibit the expression of target DNA's. For example, antisense oligonucleotides may be encapsulated which are complementary to viral sequences and utilized for antiviral treatments, e.g., hepatitis, AIDS viral  
35 infection, papillomavirus infection, etc. The use of antisense oligonucleotides for genetic therapy has been reported in the literature. See Stein and Chang, *Science* 261: 1004 (1993). Also, ribozymal RNA's may be

described in U.S. patent 5,399,346, which is hereby incorporated by reference in its entirety. See also Tolstoshev, Annu. Rev. Pharmacol. Toxicol. 33:573-96 (1993), for a general review of gene therapy, which is also incorporated herein by reference in its entirety.

The subject nucleic acid containing liposomes will, in general, be made by a method comprising:

- (i) forming a lipid film under reduced pressure,
- (ii) hydrating the lipid film by the addition of an effective amount of a nucleic acid containing solution;
- (iii) incubating the mixture for an effective time period at reduced temperature;
- (iv) adding a phosphate buffered solution to the hydrated lipid film and vortexing;
- (v) incubating at ambient temperature for a time sufficient to facilitate swelling; and
- (vi) vortexing the resultant swelled composition to produce nucleic acid containing liposomes.

Typically, there will be an intervening step between step (iv) and step (v) wherein additional phosphate buffered saline solution is added and the mixture is again vortexed. Also, after step (vi), the unencapsulated (free) nucleic acids typically will be removed. This may be effected, for example, by washing the liposomes repeatedly while centrifuging in a phosphate buffered saline solution.

In general, about 20  $\mu$ mole (about 0.64 mg) of lipid is dried under reduced pressure (30mm Hg) in a 40 mm<sup>2</sup> surface of a round-bottom glass flask or tube. The resulting dry lipid film is hydrated with about 1.4  $\mu$ l of aqueous solution containing DNA to be encapsulated. Generally, a container having a mean surface area of about 10 to 200 mm<sup>2</sup> is used, under a reduced pressure of about 1 to 50mm Hg, and a hydration volume of about 0.7 to 2  $\mu$ l.

Step (i) typically will be effected by forming a lipid film using any lipid or mixture of suitable lipids which are evaporated under a vacuum. The lipid mixture

encapsulation. In a preferred embodiment the encapsulation efficiency will be preferably at least 50%, more preferably 70%, and still more preferably will be in the range from about 70% to about 90%

5 Preferred lipids include dimyristoyl phosphatidyl glycerol, cardiolipin, phosphatidyl choline, phosphatidyl glycerol, sphingomyelin, and cholesterol. In a particularly preferred embodiment, the lipids used to make the lipid film comprise a mixture of  
10 dimyristoyldiglycerol, phosphatidylethanolamine and cholesterol. Most preferably, the molar ratio of these lipids is about 5:5:7 of dimyristoyldiglycerol, phosphatidylethanolamine and cholesterol, respectively.

Step (ii), the hydration step, in general, comprises  
15 the addition of an effective amount of a nucleic acid-containing aqueous solution to a dried lipid film. Preferably, this solution will comprise a highly concentrated, aqueous, nucleic acid solution and, more preferably, a concentrated DNA containing solution. A  
20 preferred concentration range for the DNA solution is from about 1 mg/ml to about 5 mg/ml, but concentrations outside this range may also be used, for example from about 0.01 mg/ml to about 20 mg/ml, or up until the DNA solution is saturated. In the examples, the solution  
25 comprises plasmids in an aqueous solution at a concentration of about 1-2 mg/ml. This concentrated nucleic acid solution is added to the dry lipid film in an amount sufficient to provide hydration and to achieve the desired amount of nucleic acid encapsulation.

30 For example, in one instance, a 2 mg/ml DNA plasmid solution was utilized, at 1.6  $\mu$ l solution/mg lipid, to facilitate hydration. This amount may be varied as needed. A suitable amount of the nucleic acid-containing solution will vary from about 1.7 to 17  $\mu$ g of DNA per mg  
35 of lipid if utilizing a nucleic acid solution having a concentration of about 1-5 mg/ml of DNA.

As discussed *supra*, the nucleic acid contained in this solution may comprise DNA, RNA or a mixture thereof,



colony stimulating factor-1, endothelial cell growth factor, erythropoietin, acidic and basic, fibroblast growth factor, hepatocyte growth factor, heparin binding EGF-like growth factor, insulin, insulin-like growth factors I and II, interferons  $\alpha$ ,  $\beta$ , and  $\gamma$ , keratinocyte growth factor, macrophage inflammatory protein  $\alpha$  and  $\beta$ , midkine, oncostatin M, RANTES, stem cell factor, transforming growth factors  $\alpha$  and  $\beta$ , and vascular endothelial growth factor. Examples of cell adhesion molecules include integrins, cadherins, selectins, and adhesion molecules of the immunoglobulin superfamily, such as VCAM, ICAM, PECAM, and NCAM. Examples of tumor suppressor genes include p53, DCC, Rb, and MTS1. Those of skill in the art will recognize that other genes can also be used in the invention.

In addition, as discussed *supra*, the DNA construct will contain regulatory elements that can control replication of the construct within the cell, as well as transcription and translation of genes encoded on the construct. For use in *in vivo* gene therapy it is sometimes useful for these regulatory elements to be tissue specific. The term "tissue-specific promoter" or "tissue-specific transcriptional regulatory sequence" or indicates a transcriptional regulatory sequence, promoter and / or enhancer that is induced selectively or at a higher level in cells of the target tissue than in other cells. For example, tumor cell-specific promoters include promoters that are induced selectively or at a higher level in a particular cell type or a tumor cell. Tissue specific promoters are known in the art. Examples include: the alpha-actin promoter (Shani, *Mol. Cell. Biol.*, 6:2624 (1986)); the elastase promoter (Swift et al., *Cell*, 38:639 (1984)); the alpha-fetoprotein promoter (Krumlauf et al., *Nature*, 319:224-226 (1985)); the beta-globin promoter, (Townes et al., *EMBO J.*, 4:1715 (1985)); the human growth hormone promoter (Behringer et al., *Genes Dev.*, 2:453 (1988)); the insulin promoter (Selden et al., *Nature*, 321:545 (1986)) and a prostate-specific

liposomes containing larger DNA constructs, which degrade rapidly under sonication conditions. It has been found, unexpectedly, that thorough mixing, homogenization and resuspension can be achieved by vortexing the liposome mixture.

As discussed *supra*, after the nucleic acid-containing liposomes are produced, the composition preferably will be treated to remove the free nucleic acids. This may be effected by any suitable method which does not adversely affect the liposomes, for example, by washing the liposomes in a suitable solution, e.g., phosphate buffered saline, followed by centrifugation.

Nucleic acid-containing liposomes produced in this manner may be used immediately or may be stored under favorable conditions, e.g., at about 4°C. Liposomes according to the present invention are stable for up to three weeks.

After the liposomes are produced, the encapsulation efficiency may be ascertained by known methods. For example, a DNA sample containing radiolabeled plasmid DNA constructs can be employed. This permits determination of the relative amounts of liposome-contained and free radioactivity.

The subject method reliably provides for nucleic acid entrapment efficiencies ranging from at least 25 to 50%, and more typically about 70 to 90% based upon the initial amount of nucleic acid contained in the sample, e.g., a DNA plasmid containing sample.

These liposomes may be used for both *in vitro* or *in vivo* transfection of nucleic acids into targeted cells. The targeted cells can be any cell whose cellular membrane is comprised of a lipid bilayer, and in general will comprise eukaryotic cells, and preferably mammalian cells, more preferably murine or human cells.

If the subject liposomes are to be administered *in vivo*, it may be preferable to conjugate these liposomes to a moiety which provides for the liposome to bind to targeted cells. Examples of such targeting moieties will

genetic therapy. Another variation on *in vivo* use is for the generation of genetic defects, e.g., transgenic or "knock-out" mice which are useful in the study of disease. An example of genetic therapy in a patient is  
5 when a DNA construct encoding human leukocyte antigen B7 (HLAB7) is encapsulated in a liposome as described *supra* and injected directly into the tumor lesions of a patient suffering from cutaneous melanoma, as described in Nabel et al., *Proc. Natl. Acad. Sci.* 90:11307 (1993), which is  
10 hereby incorporated by reference in its entirety. The HLAB7 stimulates the host immune response against the melanoma cells.

Generally, an *in vivo* liposomal dosage will range from about 0.2 to 20 mg/kg of body weight, and preferably  
15 from about 2 mg to 10 mg/kg of body weight. The amount will, of course, depend on the particular genetic defect, the type of nucleic acid encapsulated, the desired level of gene expression, the amount of nucleic acid contained in the liposomes, and other factors as discussed *supra*.

20 A particular advantage of the present invention is that it provides for the delivery of episomal elements to targeted cells, e.g., DNA plasmids. Preferably, the liposomes will be conjugated to a targeting moiety, e.g., an antibody to enhance delivery, when the desired target  
25 is an *in vivo* one, e.g., a tumor. This will avoid some of the problems which occur via integration of heterologous nucleic acids into normal host cell genomes.

Liposome compositions according to the present invention may be administered with additional substances,  
30 e.g., pharmaceutical carriers and excipients. Suitable carriers or excipients are described in REMINGTON'S PHARMACEUTICAL SCIENCES: DRUG RECEPTORS AND RECEPTOR THEORY, (18th ed.), Mack Publishing Co., Easton PA (1990). The choice of carrier, diluent, excipient, etc.  
35 will depend upon the desired mode of delivery. The liposomal compositions of the invention can be administered by any route currently known for delivering polynucleotide molecules to cells, including, but not

phosphatidylethanolamine, and cholesterol were mixed at a 5:5:7 molar ratio in a round-bottom flask, with a total weight of 6.4 mg. A thin lipid film was formed by rotary evaporation under vacuum. The resultant dry lipid film was then hydrated by adding 10  $\mu$ l of a 2 mg/ml solution of  $^{32}$ P-labeled plasmid DNA. The hydrated lipid film was then incubated overnight at 4°C. After this incubation period, 10  $\mu$ l of phosphate buffer saline (PBS) were added to the hydrated lipid film and the mixture was vortexed. After vortexing, supplemental PBS was added, specifically 1.4  $\mu$ l/mg lipid of PBS, and the mixture was vigorously vortexed. This vortexed composition was then allowed to swell for 2 hours at room temperature and the resulting liposome containing suspension was vortexed. On some occasions, additional PBS (8 $\mu$ l/mg of lipid) was then added if necessary. Free (unencapsulated) DNA was then removed by washing the liposomes by centrifugation (3X at 70,000 g for 30 minutes) in PBS.

The encapsulation efficiency was then determined by counting the radioactivity comprised in an aliquot of a liposomal DNA preparation. The entrapment rate was found to be 70-90% based upon the initial counts. Moreover, this result was reliably reproducible in additional trials.

EXAMPLE 2: In vitro Transfection of Mammalian Cells Using Liposomally Encapsulated DNA Plasmids

Squamous carcinoma SCC 35 cells were transfected with plasmids pSV40neo and pRSVcat which contain the neomycin resistance gene and the chloramphenicol acyltransferase gene, respectively. These plasmids were chosen to allow measurement of the efficiencies of transient and stable transfection of cells transfected by various transfection methods.

pSV40neo (Promega Corp., Madison, WI) contains a gene for neomycin resistance, thereby allowing transfected cells that express the gene product to survive in culture in medium containing neomycin. When the culture is maintained long-term in neomycin, the only cells that

These results demonstrate that nucleic acids encapsulated in liposomes according to the present invention provide for greatly enhanced transfection efficiencies relative to other available techniques, e.g., DEAE-dextran, calcium phosphate and Lipofectin™ reagent mediated transfection. While not wishing to be bound by any theory of mechanism of action, the present inventors believe that the enhanced transfection efficiencies seen with the method of the present invention are attributable to its high encapsulation efficiency, which provides a liposome population in which a very high percentage of the liposomes contain encapsulated nucleic acids.

EXAMPLE 3: Cellular Uptake of Liposomally Encapsulated Plasmid DNA

Plasmid DNA (18kb) was nick translated with <sup>35</sup>S under standard conditions. See Sambrook et al., MOLECULAR CLONING: A LABORATORY MANUAL, Second Edition, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY 1989). This DNA was then encapsulated in liposomes as described in Example 1. The liposomes were constituted from dimyristoyl phosphatidylglycerol, phosphatidylethanolamine and cholesterol in a 5:5:7 ratio, as described in Example 1. This liposomal solution was added to a culture of SCC35 squamous cell carcinoma cells seeded in microchamber glass slides, at a final liposome concentration of 1μM. The cells were then incubated for 24 h, after which they were washed and then subjected to emulsion autoradiography. This showed a dense array of black dots, corresponding to the presence of radioactively labeled plasmid DNA, in the intracellular compartment and surrounding the cell surface. These results demonstrated that liposomal DNA was effectively taken up by the cells, and penetrated

All publications and patent applications mentioned in this specification are indicative of the level of skill of those in the art to which the invention pertains. Although the foregoing refers to particular

What Is Claimed Is:

1. A method for preparing a liposome-encapsulated nucleic acid, comprising the steps of:

5 (i) incubating a hydrated lipid film for an effective time at reduced temperature, wherein said hydrated lipid film is formed by addition of a concentrated aqueous solution of a high molecular weight nucleic acid to a dried lipid film;

10 (ii) adding a minimum effective amount of phosphate buffered saline solution to the hydrated lipid film and vortexing for a time sufficient to generate liposomes and to swell said liposomes; and

(iii) vortexing the resulting swelled liposomes.

2. The method according to claim 1, wherein said  
15 lipid film comprises at least one lipid selected from the group consisting of dimyristoyl-diglycerol, phosphatidyl-ethanolamine, phosphatidylcholine, phosphatidic acid, lysophosphatidylcholine, phosphatidylserine, sphingolipids, phosphatidylglycerol, sphingomyelin,  
20 cardiolipin, glycolipids, gangliosides, cerebroside, cholesterol, tocopherol, and retinol.

3. The method according to claim 2, wherein said lipid film comprises dimyristoyldiglycerol, phosphatidyl-ethanolamine and cholesterol, in a relative molar ratio  
25 of about 5:5:7 respectively.

4. The method according to claim 1, wherein said nucleic acid solution has a concentration of 1-5 mg/ml, and wherein said nucleic acid solution is added to said dried lipid film in a ratio of less than or equal to  
30 about 1.6  $\mu$ l per mg of lipid.

5. The method according to claim 4, wherein said effective time for the incubating step is at least 12 hours.

6. The method according to claim 5, wherein said  
35 time for forming and swelling said liposomes is at least 30 minutes.

17. A liposome prepared by the method of claim 1, wherein said high molecular weight DNA encodes an antisense RNA, a ribozyme, or a therapeutic protein.

18. A method for preparing a liposome-encapsulated nucleic acid comprising the steps of:

(i) incubating a hydrated lipid film for about 2 hours at room temperature, wherein said hydrated lipid film is formed by addition of a concentrated aqueous solution of a high molecular weight nucleic acid to a dried lipid film in a ratio of less than or equal to about 1.6  $\mu$ l of DNA solution per mg of lipid;

(ii) adding a phosphate buffered saline solution to the hydrated lipid film in a ratio of less than or equal to about 1.6  $\mu$ l per mg of lipid, and vortexing for a time sufficient to generate liposomes and to swell said liposomes; and

(iii) vortexing the resulting swelled liposomes, whereby at least 25% of said nucleic acid is incorporated in said liposomes.

19. A liposome according to claim 17, wherein said therapeutic protein is selected from the group consisting of platelet-derived growth factor, epidermal growth factor, interleukins 1-14, granulocyte colony stimulating factor, granulocyte-macrophage colony stimulating factor, tumor necrosis factor, leukemia inhibitory factor, amphiregulin, angiogenin, betacellulin, calcitonin, ciliary neurotrophic factor, brain-derived neurotrophic factor, neurotrophins 3 and 4, nerve growth factor, colony stimulating factor-1, endothelial cell growth factor, erythropoietin, acidic and basic, fibroblast growth factor, hepatocyte growth factor, heparin binding EGF-like growth factor, insulin, insulin-like growth factors I and II, interferons  $\alpha$ ,  $\delta$ , and  $\gamma$ , keratinocyte growth factor, macrophage inflammatory protein  $\alpha$  and  $\delta$ , midkine, oncostatin M, RANTES, stem cell factor, transforming growth factors  $\alpha$  and  $\delta$ , and vascular endothelial growth factor.

## INTERNATIONAL SEARCH REPORT

International application No.

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## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A61K 9/127

US CL : 424/450; 935/54

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/450; 935/54

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched  
NONEElectronic data base consulted during the international search (name of data base and, where practicable, search terms used)  
NONE

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	PINNADUWAGE et al. Use of quaternary ammonium detergent in liposome mediated DNA transfection of mouse L-cells. Biochemica et Biophysica Acta. 1989, Vol. 985, pages 33-37, especially page 34.	1-20
Y	US 4,902,512 A (ISHIGAMI ET AL.) 20 February 1990 (20.02.90), column 4, lines 25-47.	1-20
Y	US 4,762,720 A (JIZOMOTO) 09 August 1988 (09.08.88), column 6, lines 13-39.	1-20

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

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